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Original Article

Alteration of apoptotic protease activating factor 1 expression and possible role in $ONOO^-$-induced apoptosis in human cerebral vascular smooth muscle cells

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Abstract: The study was to investigate the change of apoptotic protease activating factor 1 (Apaf1) expression in the human cerebral vascular smooth muscle cells (HCVSMCs) in response to peroxynitrite ($ONOO^-$). HCVSMCs were cultured in vitro and $ONOO^-$ of different concentrations was directly added to the culture medium. The total proteins were extracted and the alteration of Apaf1 expression was examined by the means of Western-blot. Apaf-1 siRNA (h) was put into another plate of HCVSMCs for transfection. The transfected cells were also incubated in different concentrations of $ONOO^-$. The change of Apaf1 protein expression after siRNA transfection was examined by the means of Western-blot. The morphological changes were observed by acridine orange staining to determine whether cells experienced apoptosis in response to $ONOO^-$ before and after siRNA. The Flow cytometry analysis was used to examine the change of cells apoptotic rates in response to $ONOO^-$ before and after siRNA. Obviously, there was up-regulated Apaf1 expression at protein level in the course of HCVSMCs apoptosis induced by $ONOO^-$. When Apaf1 expression was suppressed, the apoptotic sum of HCVSMCs didn't change. This study demonstrates that Apaf1 gene is involved in $ONOO^-$-induced apoptosis in HCVSMCs. Whether HCVSMCs treated by $ONOO^-$ undergo apoptosis depends on Apaf1 level.

Keywords: $ONOO^-$, apoptotic protease activating factor 1, human cerebral vascular smooth muscle cell, apoptosis

Introduction

In the general physiological conditions, nitric oxide (NO) is a pivotal factor in regulating the function of blood vessel. It has been reported that NO could induce apoptosis of different types of cells, such as macrophages, chondrocytes, cardiac myocytes, and smooth muscle cells, but it seems that NO leads to contrastive effects depending on the concentration of NO. A low concentration of NO has the anti-apoptotic effect, while relatively higher concentration of NO induces apoptosis in vascular smooth muscle cells (VSMCs) [1]. The overabundance of endogenous NO has cytotoxic effect in the pathological conditions, which is mainly due to the production of free radicals caused by Peroxynitrite ($ONOO^-$) [2, 3]. $ONOO^-$, the reaction product of NO and superoxide anion, is involved in a series of pathophysiological processes in the human body. In the cardiovascular system, $ONOO^-$ directly regulates the proliferation, migration and gene expression of vascular smooth muscle cell, and indirectly modifies the signal transduction of other cytokines in the vascular smooth muscle cell. $ONOO^-$ can attack biological molecules in the vascular endothelial cells, VSMCs and cardiac muscle cells as a strong oxidizer, and lead to cardiovascular dysfunction [4]. Zou, et al firstly purified apoptotic protease activating factor 1 (Apaf1) cDNA from the endochylema of HeLa cells and cloned it in 1999 [5]. Apaf1 plays an important role in apoptosis mechanism mediated by mitochondria. Different biological signals promote mitochondria to release cytochrome C.

Cytochrome C combines with Apaf1 in the presence of dATP/ATP, launches the caspase cascade, and cracks nucleoprotein, cytoskeleton, endoplasmic reticulum, which causes the typical morphological changes of apoptosis [6]. Apaf1 is widely distributed in various tissues and cells, such as the brain, heart, liver, spleen, lung, pancreas, and peripheral blood leukocyte [7]. Higher Apaf1 gene expression in adult spleen, peripheral white blood cells, embryo brain, embryo lung and kidney is consistent with high apoptosis level in these tissues [8].

$ONOO^-$ can also induce apoptosis in vascular smooth muscle cells of canine and rat brain [9]. However, the precise mechanism on how $ONOO^-$ causes apoptosis in human central vascular smooth muscle cells (HCVSMCs) is still unclear. So far, the research reports about the effect of $ONOO^-$ on the HCVSMCs biological characteristics, especially apoptosis are scanty. And it's also unclear whether Apaf1 expression in HCVSMCs is associated with apoptosis caused by $ONOO^-$. Therefore, we designed this research to determine whether HCVSMCs undergo apoptosis after treated by $ONOO^-$ and whether the apoptotic process concerns Apaf1.

Material and methods

Materials

The HCVSMC line, smooth muscle cell medium for definite purpose, fetal bovine serum and growing factors of smooth muscle cells were purchased from the ScienCell Research Laboratories (USA). $ONOO^-$ was purchased from Cayman CHEMICAL (USA, Cat. 81565) and frozen at -80°C before use since the product is heat and light sensitive. Activity decreases approximately 2% per day at -20°C. Apaf-1 siRNA (h) (sc-29201) was purchased from Santa Cruz Biotechnology, Inc. Apaf-1-ALT (H324) pAb and β-Actin (D8) pAb were purchased from Bioworld, Technology, Inc.

Cell culture and treatment with $ONOO^-$

The HCVSMCs were cultured in smooth muscle cells medium containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere composed of 95% air and 5% CO_2. Cells were periodically sub-cultured with 0.25% trypsin and seeded into 24 or 96-well plate for different experiments. Previously, it had been proved by MTT assay that $ONOO^-$ led to the apparent reduction of HCVSMCs viability within 24 hours in a concentration-dependent manner [10]. Thus, we maintained the culture medium at final $ONOO^-$ concentrations of 10, 50, 100 μM for 24 hours of incubation in the present study. Each cell line (1×10^4 cells) was seeded in 96-well dishes containing 100 μl culture medium per well. After 24 hours of incubation, the medium was removed and refilled with new serum-free medium for 24 hours of incubation.

Protein extraction and western blot analysis

The cell line was washed once with PBS. HCVSMCs were lysed in 300 μl 1× electrophoresis sample buffer by gently rocking the 6-well plates. The lysate was sonicated on ice. The supernatant was located at -80°C until use. The Bradford method was applied to determine the protein concentration of the supernatant. 40 μg sample was boiled at 95°C for 5 minutes and loaded onto sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 5% stacking gel and 15% separating gel), which was followed by a separation at 80 V for 2 hours and subsequent transfer to a polyvinylidene difluoride membrane. PVDF membrane was blocked in 5% defatted milk for 1 hour at room temperature, incubated in the first antibodies [(Apaf1 1:1000, Rabbit anti-Human); (β-actin, 1:2000, mouse anti-human)], and diluted in 5% defatted milk/Tris-buffered saline-Tween (TBST) overnight at 4°C. After washed by TBST, the membrane was incubated with a secondary antibody against mouse IgG accordingly and the signals were observed in ECL. The Apaf1 and β-actin bands could be observed at apparent molecular weights of 38 and 42 kDa. Relative OD ratio was calculated with NIH software Image J by comparison with β-actin from 3 experiments.

siRNA preparation and transfection

Apaf-1 siRNA (h), a target-specific 19-25 nt siRNA, was designed to knock down gene expression, which was recommended for the inhibition of Apaf-1 expression in human cells. Resuspended lyophilized siRNA duplex in 330 μl of the RNAse-free water was provided. The resuspension of siRNA duplex and 330 μl of RNAse-free water merged into a 10 μM solution including 10 μM Tris-HCl, pH 8.0, 20 mM NaCl, and 1 mM EDTA buffered solution. In a six well

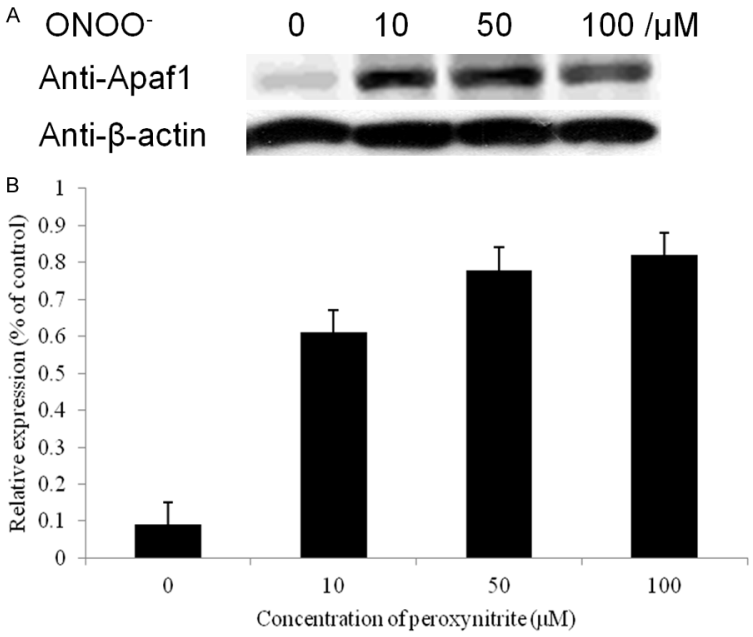

Figure 1. Effect of $ONOO^-$ on expression of Apaf1 protein in HCVSMCs. A. Western-blot analysis of Apaf1 in HCVSMCs exposed to final $ONOO^-$ concentrations of 0, 10, 50, 100 μM. B. Protein levels were quantified by densitometric analysis with β-actin as standard.

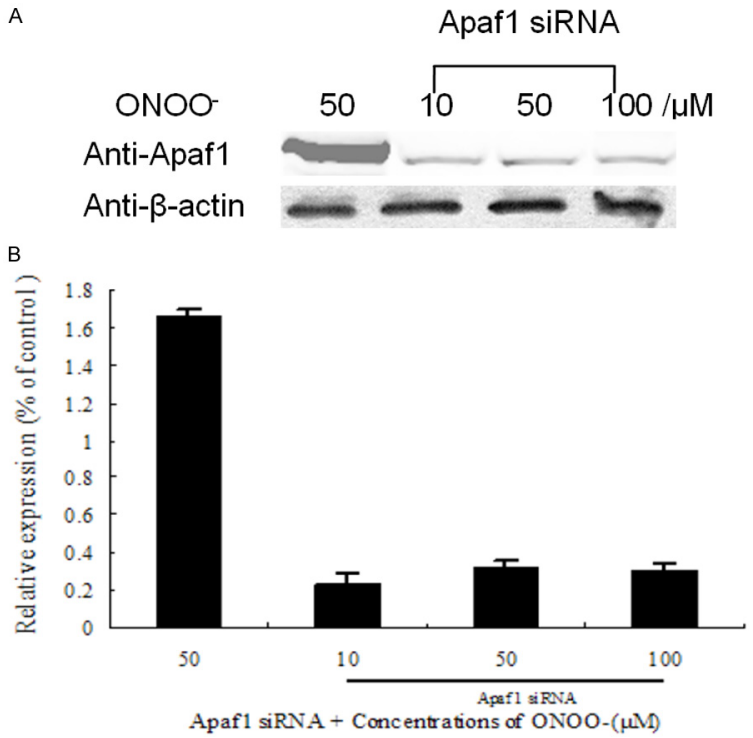

Figure 2. Inhibitory effect of Apaf1 siRNA on Apaf1 protein expression in HCVSMCs exposed to $ONOO^-$. A. Western-blot analysis of Apaf1 in HCVSMCs transfected by Apaf1 siRNA in response to $ONOO^-$ of 10, 50, 100 μM, with the untransfected HCVSMCs in response to $ONOO^-$ of 50 μM as the control group. B. Protein levels were quantified by densitometric analysis with β-actin as standard.

tissue culture plate, 2×10^5 cells were seeded per well in 2 ml antibiotic-free normal growth medium supplemented with FBS. The cells were incubated at 37°C in a CO_2 incubator until the 60~80% cells were confluent. This process usually took 24 hours. The following solutions were compounded, such as Solution A [5 μl siRNA duplex (i.e. 0.5 μg siRNA) was diluted into 100 μl siRNA Transfection Medium for each transfection] and Solution B [6 μl siRNA Transfection Reagent was diluted into 100 μl siRNA Transfection Medium for each transfection]. The Solution A was added directly to Solution B with a pipette. The solutions were mixed gently up and down and incubated for 30 minutes at room temperature. The cells were washed once with 2 ml of siRNA Transfection Medium. The medium was aspirated off. For each transfection, 0.8 ml siRNA Transfection Medium was added to each tube containing the siRNA transfection reagent mixture (Solution A + Solution B). The mixture was mixed gently and overlaid onto the washed cells. The cells were incubated for 6 hours at 37°C in a CO_2 incubator. 1 ml of normal growth medium was added which containing 2 times normal serum and antibiotics concentration (2× normal growth medium) without removing the transfection mixture. The cells were incubated for an additional 24 hours. The medium was aspirated off and replaced with fresh 1× normal growth medium.

Protein extraction of transfected cells and western blot analysis

HCVSMCs transfected by Apaf-1 siRNA (h) (5×10^4/mL) were

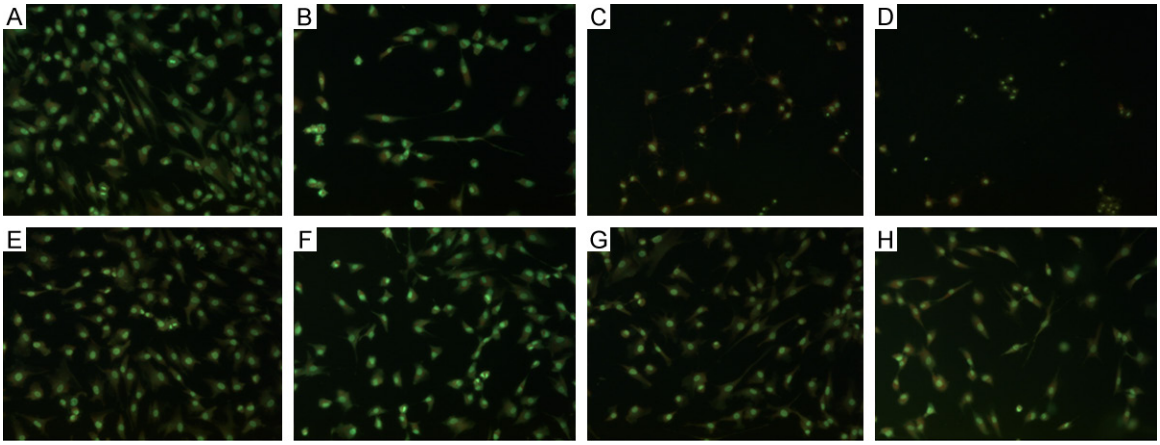

Figure 3. Morphological changes in HCVSMCs treated with $ONOO^-$ by acridine orange under fluorescence microscopy (×50). A. 0 μM. B. 10 μM. C. 50 μM. D. 100 μM. E. 0 μM with Apaf1 siRNA. F. 10 μM with Apaf1 siRNA. G. 50 μM with Apaf1 siRNA. H. 100 μM with Apaf1 siRNA.

seeded into 6-well dishes and treated with final $ONOO^-$ concentrations of 10, 50, 100 μM for 24 hours. The total proteins were extracted and incubated in the first antibodies [(Apaf1 1:1000, Rabbit anti-Human); (β-actin, 1:2000, mouse anti-human)] with the method mentioned above.

Acridine orange cytochemistry staining

Each cell line (5×10^4 cells) was seeded in 24-well dishes containing 1 ml culture medium. After 24 hours of incubation, the medium was removed and refilled with new serum-free medium containing final $ONOO^-$ concentrations of 0, 10, 50, 100 μM for 24 hours incubation. The cells were washed twice by PBS, then fixed by 95% alcohol for 10 minutes, and stained by 0.01% acridine orange for 5 minutes. Morphological change was observed by fluorescence microscope. The same method was used to observe the morphological change of HCVSMCs treated by Apaf1 siRNA transfection.

Flow cytometry analysis

Cell apoptotic process was assessed by flow cytometry through Annexin V-FITC and propidium iodide (PI) staining. Briefly, cells (5×10^4/mL) were seeded into 6-well dishes and treated with final $ONOO^-$ concentrations of 0, 10, 50, 100 μM. After the treatment, cells were trypsinized and washed with phosphate-buffered saline (PBS). After being washed, cells (1×10^7/mL) were resuspended in binding buffer (200 μl) containing Annexin V-FITC (5 μl, 20 μg/ml) and PI (10 μl, 20 μg/ml) for 15 minutes at room temperature, and then Binding Buffer (300 μl) was added before analysis with a FACScan (BectonDickinson, Mountain View, CA, USA) by Cell Quest software (BD Biosciences, San Jose, CA, USA). The same method was used to examine the apoptotic rate of HCVSMCs treated by Apaf1 siRNA transfection.

Statistic analysis

Data were presented as mean ± standard error on the mean (SEM). Statistical calculations were performed in SPSS14.0 software package. Analysis of Variance (ANOVA) was used. *P* values of less than 0.05 were considered significant.

Results

Effects of $ONOO^-$ on Apaf1 expression in HCVSMCs by western-blot

In comparison with untreated cells, the expressions of Apaf1 in three experimental groups in 24 hours (10, 50, 100 μM concentrations of $ONOO^-$) were up-regulated with β-actin as a standard. Image J software was used to analyze the relative photodensity, with β-actin as a standard. Statistic analysis showed that Apaf1 protein expressions in groups treated by $ONOO^-$ were significantly higher than that in the control group in the concentration-dependent manner ($P<0.05$) (**Figure 1**).

Expression of Apaf1 was inhibited after siRNA transfection

The total protein extracted from the HCVSMCs was transfected by Apaf1 siRNA and western

Alteration of Apaf1 in $ONOO^-$-induced apoptosis in HCVSMCs

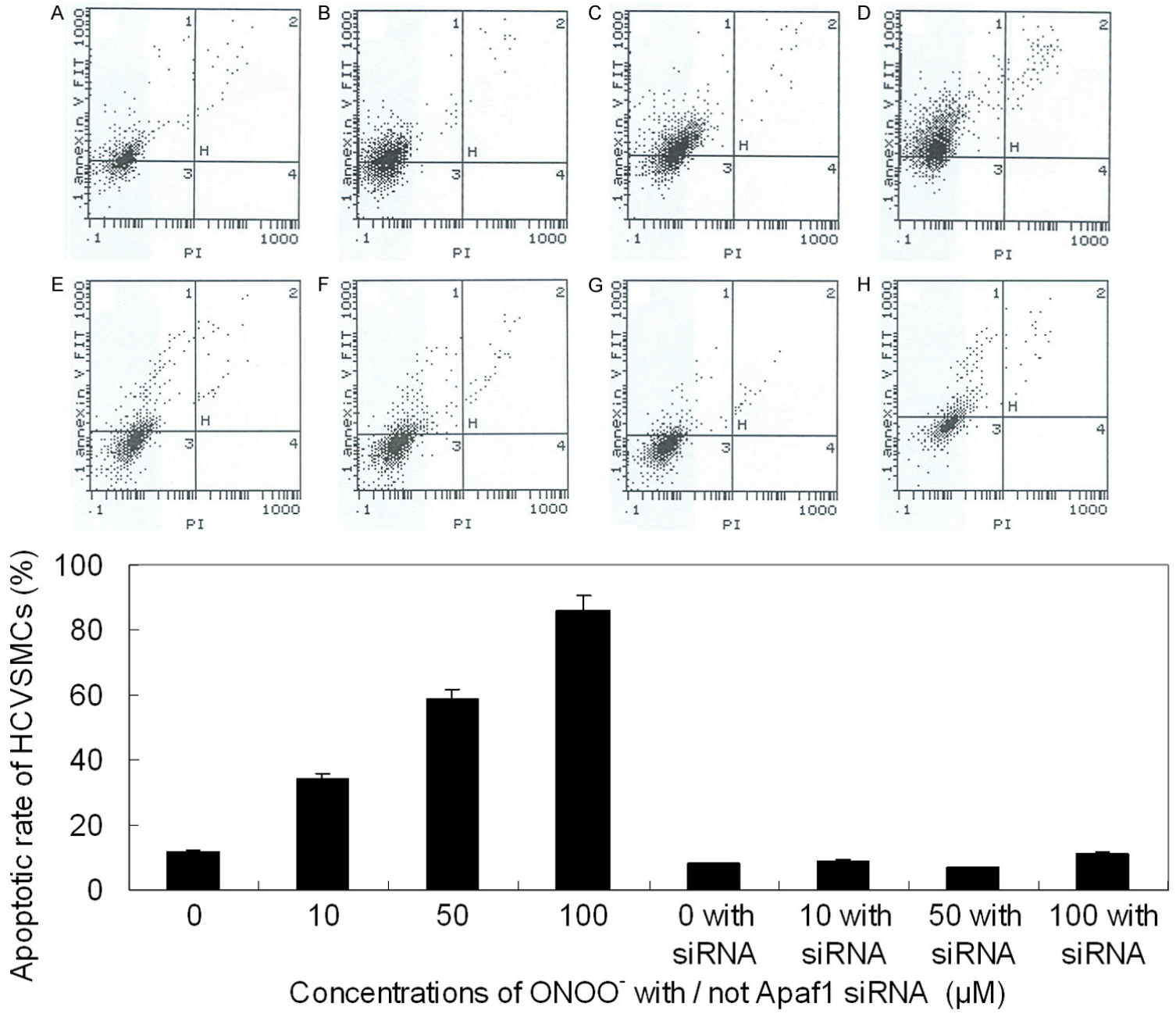

Figure 4. Flow cytometry analysis of HCVSMCs treated with $ONOO^-$. A. 0 μM. B. 10 μM. C. 50 μM. D. 100 μM. E. 0 μM with Apaf1 siRNA. F. 10 μM with Apaf1 siRNA. G. 50 μM with Apaf1 siRNA. H. 100 μM with Apaf1 siRNA. Apoptotic rates of HCVSMCs getting from flow cytometry were analyzed by analysis of variance.

blot analysis was conducted. Compared with cells only treated by 50 μM concentrations of $ONOO^-$, Apaf1 expressions of 3 groups with siRNA transfection after incubation in 10, 50, 100 μM concentrations of $ONOO^-$ sharply reduced ($P<0.05$) (**Figure 2**).

The apoptotic rate of HCVSMCs with Apaf1 siRNA didn't change when they were treated by the same concentration of $ONOO^-$

Acridine orange cytochemistry staining result showed that when the level of Apaf1 in transfected HCVSMCs treated by $ONOO^-$ was very low, there was no morphological apoptotic change in comparison with cells untreated by siRNA yet in the same concentration of $ONOO^-$ (**Figure 3**). Flow cytometry analysis proved that apoptotic rates of transfected HCVSMCs didn't change in comparison with cells untreated by siRNA when they were incubated in the same concentration of $ONOO^-$ (**Figure 4**).

Discussion

Reactive nitrogen species (RNS) play a pivotal role in diverse types of vascular-related diseases, i.e., hyper-tension, atherosclerosis [11, 12]. $ONOO^-$ is a representative chemical molecule of RNS. Previous research demonstrated that $ONOO^-$ could pass through phospholipid membrane bilayers freely to arrive the domain which is full of a series of molecular targets, leading to cell death via necrosis or apoptosis [11, 13]. *In vitro* studies proved that the response elicited by exposure of cells to $ONOO^-$ depended on $ONOO^-$ concentration and cell types, including VSMCs [14]. Since Salgo et al firstly found that $ONOO^-$ could induce apoptosis in thymocytes [15], a great number of researches demonstrated that $ONOO^-$ could also induce apoptosis in different cell lines in vitro [16]. However, the biological mechanisms of how $ONOO^-$ induces apoptosis in VSMCs have been studied sparingly in VSMCs line, so the role of Apaf1 playing in vascular diseases isn't clear. Because Apaf1 is greatly concerned with cell apoptosis, we assume that there is a relation between the level of Apaf1 and the course of $ONOO^-$-induced apoptosis in HCVSMCs.

In the present study, we demonstrated that the expression of Apaf1 protein in HCVSMCs increased in response to high concentration of $ONOO^-$. This result is similar to previous reports on tumor cells [17]. Our result implies that $ONOO^-$-induced apoptosis of HCVSMCs is related to Apaf1 expression. When Apaf1 expression is restrained obviously by Apaf1 siRNA transfection, there aren't apparent apoptotic changes of HCVSMCs, compared with HCVSMCs without Apaf1 siRNA transfection, which implies that Apaf1 low level is related to normal cell survival rate. The morphological change and flow cytometry analysis both demonstrated that whether HCVSMCs treated by $ONOO^-$ undergo apoptosis completely lay in Apaf1 expression. Therefore, we assume that Apaf1 directly takes part in the $ONOO^-$-induced apoptotic process in HCVSMCs.

Two pivotal apoptotic pathways, the mitochondrial pathway and the death receptor pathway, dominate the cell apoptotic process. These pathways activate the expression of caspase cascades through a few of interactions [18]. Apoptosis is adjusted by different apoptosis-related proteins via these two pathways, so how Apaf1 is involved in these two pathways needs further study.

Conclusions

To sum up, our results show that Apaf1 participates in $ONOO^-$-induced apoptosis in HCVSMCs. Whether HCVSMCs treated by $ONOO^-$ undergo apoptosis depends on Apaf1 level. $ONOO^-$ production in vivo is an important pathogenic mechanism of vascular diseases, so the research aiming at reducing $ONOO^-$ will provide a new therapeutic approach for vascular disorders in the future.

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Disclosure of conflict of interest

None.

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